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The Amino Acid Composition of Crystalline Beef Trypsin*)

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(Received June 3, 1964)

INTRODUCTION

In the course of our study on crystallized trypsin and its degradation products the necessity for a complete amino acid analysis of this enzyme was felt. However, the data on the quantitative amino acid composition of crystalline beef trypsin and trypsinogen reported in the litterature [1–8] is sometimes incomplete and in many cases varies considerably. For these reasons we undertook the present work which deals with the analysis of the crystallized trypsin produced by the Novo Industri and which is currently employed in our studies.

EXPERIMENTAL

MATERIAL: Trypsin was purchased from *Novo Industri,* batch 114–3, Copenhagen, Denmark.

N-bromosuccinimide (Matheson, Coleman & Bell) was used without further purification.

Oxidation of trypsin was performed with performic acid prepared from 30% hydrogen peroxide (Merck) and 88% formic acid in a ratio 1:9 [9]. Dialysis was carried out with cellophane bags from the Visking Company.

Standard amino acids were purchased from the California Corporation for Biochemical Research and were chromatographically pure. Ninhydrin, hydrindantin, methyl cellosolve, thiodiglycol and BRIJ–35 were purchased from the Pierce Chemical Corporation and were used without further purification.

All other reagents were commercial preparations of analytical purity and were employed in the original form.

METHODS

MOISTURE AND TOTAL NITROGEN: The protein was equilibrated with air and duplicate samples were taken for moisture and total nitrogen determinations. Moisture was determined in a vacuum over P_2O_5 at 100°C. Total nitrogen was determined by the micro-Kjeldahl method [21].

*) This work has been supported in part by grants from the *Superintendência do Desenvolvimento do Nordeste,* the *Conselho Nacional de Pesquisas* and the *Foreign Research and Technical Programs Division, Agricultural Research Service* of the *U. S. Department of Agriculture* (FG–Br–100).

DETERMINATION OF THE EXTINCTION COEFFICIENT: The extinction coefficient at 280 mμ was calculated from measurements of the optical density of several dilutions of a stock solution prepared by dissolving a known amount of dried trypsin in 0,0025N HCl. It was found to be 1,61/cm/mg/ml.

All spectrophotometric determinations were carried out by measuring absorbances in a Beckman DU Spectrophotometer using quartz cells with 1 cm light path.

DETERMINATION OF TRYPTOPHAN: It was determined by two separate and independent methods:

a) *Spectrophotometric method of Goodwin and Morton* [10]: The spectrophotometric estimation of tryptophan was carried out by measuring the absorbance at 280 and 294,4 mμ of a solution containing 1,5 mg of trypsin dissolved in 2,5 ml of 0,1 N HONa. The tryptophan content was calculated assuming 10 tyrosine residues per trypsin mol as given by the amino acid analysis.

b) *Oxidation with NBS* [11–13]: 0,035%, 0,04% and 0,055% trypsin solutions in sodium acetate buffer 0,1M, pH 4,0 were prepared. These trypsin solutions were titrated respectively with 0,5 mM, 0,05mM and 0,005mM aqueous solutions of NBS. A 2 ml aliquot of each trypsin solution was placed in a quartz cuvette and small aliquots of the respective NBS solution were added. Through mixing was carried out by blowing a stream of small bubbles through a thin cappilary tube. The absorbance at 280 mμ was recorded after each NBS addition. After correction for dilution the maximum decrease in absorbance for each concentration was obtained. A tryptophan molar extinction coefficient of 5500 and a correction factor of 1,31 were employed in the calculation of the tryptophan content.

DETERMINATION OF CYSTINE: The cystine content was chromatographically determined as cysteic acid on a 30 hours hydrolysate of performic acid oxidized trypsin by the method of SCHRAM [9]. Oxidation was carried out by dissolving 24 mg of trypsin in 25 ml of performic acid solution at 0°C. The reaction was allowed to proceed for 4 hours at the same temperature and stopped by addition of a twofold excess of distilled water. The solution was dialyzed in cellpohane bags against 0,2N acetic acid and then freeze-dried. The resulting protein was hydrolyzed for 30 hours in standard conditions.

HYDROLYSIS: One part of protein was hydrolysed with 1000 parts of redistilled constant boiling HCl in neutral glass tubes sealed under vacuum. After hydrolysis for 8, 16, 30 and 70 hours each tube was cooled by immersion in a dry ice-acetone mixture and opened. The contents were immediately transferred to a Petri dish and dried over NaOH and P_2O_5 in a vacuum dessicator. The hydrolysates were then dissolved in citric acid/citrate buffer of pH 2,91, 0,25N, and a 2 ml aliquot was applied to the chromatographic column. The remaining hydrolysate solution was used for the determination of nitrogen by the micro-Kjeldahl method and the resulting value used to calculate the initial trypsin concentration.

AMINO ACID ANALYSIS: The hydrolysates were automatically analyzed in duplicate by the method of SPACKMAN, STEIN AND MOORE [14] as modified by PIEZ AND MORRIS [15] using a *Technicon Amino Acid Analizer* (Technicon Chromatography Corporation, Chauncey, N. Y., U.S.A.).

TABLE III

Reported Amino Acid Analyses of Beef Trypsin

AMINO ACID	RESIDUES/TRYPSIN MOL								
	1	2 (*)	3	4	5 (*)	6	7 (*)	8	This paper
Aspartic	20	21	20	22	20	19	22	22	21
Threonine	9—11	11	8	10	9—11	8	11	11	10
Serine	38	—	26	32	38	26	40	38	32
Glutamic	10	17	9	14	10	12	11	15	14
Proline	7	12	8	8	7	8	14—15	8	9
Glycine	21	—	20	25	21	20	—	25	25
Alanine	13	16	10	14	13	12	—	15	14
1/2 Cystine	—	13	10	12	11—13	—	12	12	12
Valine	14	17	8	12	14	13	—	16	14
Methionine	1	2	1	1	1	2	—	2	2
Isoleucine	12	} 33	9	12	12	12	—	14	14
Leucine	12		11	14	12	12	—	14	15
Tyrosine	9	8	5	9	9	8	4	10	10
Phenylalanine	4	6	2	3	4	3	3	3	3
Ammonia	23±3	—	—	29	23±3	—	—	29	29
Lysine	13	16	9	14	13	11	13—14	14	16
Histidine	3	2	2	3	3	3	3	3	3
Arginine	2	2	2	2	2	2	2	2	2
Tryptophan	—	4	1	4	—	5	—	4	4
HO-lysine	—	—	—	—	—	—	—	1	—
TOTAL (**)	189±1	180	166	211	201±2	176	136±1	229	220

(*) Less the trypsinogen activation hexapeptide — $Val(Asp)_4Lys$.

(**) Ammonia excluded.

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per trypsin molecule by both procedures. This value is consistent with the results given by RAMACHANDRAN [13] and VISWANATHA [4] by NBS oxidation and with those of KEIL [2] by paper chromatography.

TABLE II

Amino Acid Composition of Beef Trypsin

AMINO ACID	PROTEIN		N as % of total N	NUMBER OF RESIDUES		M.W. calculated from a.a. composition
	% as amino acid	% as residue		For M.W. 23 800	Rounded off	
Aspartic	11,75	10,25	7,35	21,09	21	23 698
Threonine	5,01	4,29	3,39	9,72	10	25 586
Serine	14,13	11,85	11,23	32,22	32	23 637
Glutamic	8,65	7,65	4,78	13,71	14	24 303
Proline	4,35	3,71	3,15	9,03	9	23 721
Glycine	7,89	6,10	8,86	25,41	25	23 416
Alanine	5,24	4,24	4,91	14,07	14	23 682
1/2 Cystine	6,06	5,25	4,02	11,53	12	24 770
Valine	6,89	5,89	4,85	13,91	14	23 954
Methionine	1,25	1,11	0,65	1,87	2	25 455
Isoleucine	7,72	6,72	5,06	14,50	14	22 979
Leucine	8,27	7,20	5,16	14,80	15	24 122
Tyrosine	7,61	6,90	3,61	10,34	10	23 017
Phenylalanine	2,08	1,87	1,07	3,07	3	23 257
Lysine	9,83	8,69	11,49	16,47	16	23 121
Histidine	1,96	1,74	3,38	3,23	3	22 105
Arginine	1,46	1,32	3,53	2,53	2	18 814(*)
Ammonia	2,07	1,14	10,10	28,97	29	23 852
Tryptophan	3,43	3,15	2,79	—	4	—
TOTAL	115,65	99,07	99,38	246,47	220	Mean = 23 746

(*) Arginine excluded.

The maximum yield obtained for each amino acid was the value used to calculate the amino acid composition of trypsin. These data are compiled in TABLE II. On the basis of percentage composition 99,07% of the protein weight and 99,38% of the total nitrogen of 15,03% as estimated by the micro-Kjeldahl procedure are accounted for. The molecular weight of trypsin as calculated from the amino acid composition — arginine excluded — is also given in TABLE II. It agrees well with the results obtained by physico-chemical methods [20].

In TABLE III our results are compared with the values obtained by other laboratories. Our data is consistent with those given by VISWANATHA AND ZMRHAL [8]. However, significant notable differences are shown by the contents of valine, methionine, isoleucine and lysine as obtained by the former author, and valine, serine, hydroxy-lysine by the latter. Our results for valine are in agreement with those of COHEN [1] and SÖRM [5]. That of methionine with those of BUCK [6] AND KEIL [2]. Hydroxy-lisine was not detected in any hydrolysate. The value we found for lysine is the highest so far reported in the litterature for analysis by column chromatography.

RESULTS AND DISCUSSION

The data compiled in TABLE I present average results obtained after increased periods of hydrolysis. All calculations are on a dry weight basis.

TABLE I

AMINO ACID	RESIDUES/TRYPSIN MOL				
	8 h	16 h	30 h	70 h	Ox. tryspin
Cysteic	—	—	—	—	12
Aspartic	20	21	21	16	17
Threonine	8	9	10	7	8
Serine	30	32	30	22	24
Glutamic	12	14	14	10	10
Proline	8	9	9	7	6
Glycine	23	25	24	20	18
Alanine	13	14	14	11	10
1/2 Cystine	8	10	12	4	—
Valine	8	11	14	13	10
Methionine	1	2	2	1	—
Isoleucine	8	13	14	13	10
Leucine	12	14	15	11	10
Tyrosine	9	10	10	7	1
Phenylalanine	3	3	3	3	2
Ammonia	24	29	27	28	24
Lysine	13	16	15	12	10
Histidine	2	3	3	2	2
Arginine	2	2	2	2	—
TOTAL	184	212	216	161	174

The results in TABLE I show that hydrolysis for 8 hours is sufficient to give the maximum yield of aspartic acid, phenyl alanine and arginine. Most of the amino acids, such as serine, glutamic acid, proline, glycine, alanine, methionine, tyrosine, lysine and histidine required 16 hours of hydrolysis to be liberated. A longer hydrolysis time, i.e., 30 hours was, however, needed for threonine, 1/2 cystine, valine, isoleucine and leucine.

Hydrolysis for 70 hours led to destruction to a great extent of most of the amino acids except valine and isoleucine. The values for serine, threonine and tyrosine for the 30 and 70 hours hydrolysates showed decomposition of about 30%. Cystine destruction amounted to almost 70% under the conditions employed.

The increase in the amount of valine and isoleucine obtained in the 30 hours hydrolysate agrees with the observation of SYNGE concerning the stability of valyl peptides to acid hydrolysis [16] due to a steric hindrance of proton attack.

The amino acid composition of performic acid oxidized trypsin approaches that of the 70 hours hydrolysate. Tyrosine was almost completely destroyed while cystine was quantitatively transformed and recovered as 12 cysteic acid residues. This result agrees well with those obtained by CARTER [17] and CECIL AND WAKE [18] using argentometric titration of sulphide reduced trypsin and also with the value obtained by EPSTEIN AND ANFINSEN [19] using p-chloro-mercuribenzoate titration of mercaptoethanol reduced trypsin.

The tryptophan content was calculated from the results obtained by specific methods and therefore is given separately in TABLE II. It was found to be 4 residues

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